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Published in:
Biochemistry

DOI:
[10.1021/bi981532j](https://doi.org/10.1021/bi981532j)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1998

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Hildeng-Hauge, H., Mantzilas, D., Moll, G. N., Konings, W. N., Driessen, A. J. M., Eijssink, V. G. H., & Nissen-Meyer, J. (1998). Plantaricin A is an amphiphilic alpha-helical bacteriocin-like pheromone which exerts antimicrobial and pheromone activities through different mechanisms. *Biochemistry*, 37(46), 16026 - 16032. <https://doi.org/10.1021/bi981532j>

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Plantaricin A Is an Amphiphilic α -Helical Bacteriocin-like Pheromone Which Exerts Antimicrobial and Pheromone Activities through Different Mechanisms[†]

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Received June 29, 1998; Revised Manuscript Received September 1, 1998

ABSTRACT: Production of bacteriocins by lactic acid bacteria is in some cases regulated by a quorum sensing mechanism that involves a secreted bacteriocin-like peptide pheromone. In the case of *Lactobacillus plantarum* C11, this pheromone, the 26-mer plantaricin A (PlnA), has the interesting property of having both bacteriocin and pheromone activities. To gain insight into how PlnA functions as a pheromone and as a bacteriocin, the L- and D-enantiomers of an N-terminally truncated form of PlnA were synthesized (PlnA-22L and PlnA-22D; PlnA-22L has full biological activity). With circular dichroism, it was shown that the two peptides are unstructured in aqueous solution, but they adopt mirror-image amphiphilic helical structures in the presence of trifluoroethanol and membrane-mimicking entities such as micelles of dodecylphosphocholine and negatively charged Ole₂GroPGro liposomes, but not in the presence of zwitterionic Ole₂GroPCho liposomes. Thus, the negative charge on the membrane is important for structuring of the (positively charged) PlnA peptides. In terms of in vivo antimicrobial activity, PlnA-22L and PlnA-22D behaved almost identically. Likewise, the peptides dissipated the membrane potential and the transmembrane pH gradient in sensitive cells equally effectively. PlnA-22L induced bacteriocin production in *L. plantarum* C11 (i.e., displayed pheromone activity), the level of induction being clearly dose-dependent. PlnA-22D did not display pheromone activity, but, at high concentrations, was able to inhibit the pheromone activity of PlnA-22L. The results indicate that the antimicrobial activity of PlnA does not require chiral interactions and is mediated through the formation of a strongly amphiphilic α -helical structure. In contrast, PlnA's pheromone activity is dependent on a chiral interaction between the amphiphilic helix (PlnA-22L) and a receptor protein. One may speculate that PlnA is an evolutionary intermediate between a true bacteriocin and a pheromone.

Many lactic acid bacteria are known to secrete one or more antimicrobial peptides called bacteriocins (1, 2). These peptides are usually basic and between 30 and 60 residues long. The production of bacteriocins is, at least in some strains, controlled by a quorum sensing mechanism that involves a secreted peptide pheromone with bacteriocin-like characteristics (3–7). Like bacteriocins, these peptide pheromones are cationic and they are exported from the cell with help of a bacteriocin-type leader peptide and secretion machinery. In some cases, the peptide pheromones also display bacteriocin activity, whereas in other cases they act solely as a pheromone (4, 5, 8).

Anderssen et al. (8) recently showed that plantaricin A (PlnA),¹ the peptide pheromone controlling bacteriocin production in *Lactobacillus plantarum* C11, possesses strain-specific antimicrobial activity. Three PlnA variants have been identified in culture medium of *L. plantarum* C11: a 26 residue peptide (PlnA-26) and two N-terminally truncated forms containing 23 (PlnA-23) and 22 (PlnA-22) residues. The three variants are all derived from a 48-residue precursor encoded by the *plnA* gene (9), and they display identical pheromone (3) and almost identical bacteriocin activities (8).

Exposing *L. plantarum* C11 to PlnA induces the transcription of genes encoding two two-peptide bacteriocins (PlnE/F and PlnJ/K) and their immunity proteins, as well as the proteins necessary for secretion and processing of these

[†] This work was supported by the Norwegian Research Council.

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¹ Abbreviations: BCECF, 2',7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein; CD, circular dichroism; DiSC₃(5), 3,3'-dipropylthiadicarbocyanine iodide; Ole₂GroPCho, dioleoyl-L- α -phosphatidylcholine; Ole₂GroPGro, dioleoyl-L- α -phosphatidyl-DL-glycerol; Pln, plantaricin; Pln-22D, D-enantiomer of PlnA-22-mer; Pln-22L, L-enantiomer of PlnA-22-mer; $[\theta]_{222}$, mean residual ellipticity at 222 nm.

bacteriocins (3, 8, 10). PlnA also induces transcription of the *plnABCD* operon which encodes PlnA and a "two"-component regulatory system (11) consisting of a membrane-associated histidine protein kinase (PlnB) and two response regulators [PlnC and PlnD (9, 10, 12)]. Operons similar to the *plnABCD* operon, as well as peptides functionally similar to PlnA, have been detected in other bacteriocin-producing lactic acid bacteria (4, 5, 7, 13–15). It is believed that the pheromone interacts with the histidine kinase, thereby triggering its autophosphorylation and subsequent transfer of the phosphate group to the response regulator(s) (7, 11, 16). The response regulators bind to specific promoter elements, thus activating gene expression (7, 10, 12, 16).

In this study we have synthesized two enantiomeric forms of PlnA-22: one containing only L-amino acids (PlnA-22L, the naturally produced form) and one containing only D-amino acids (PlnA-22D). We have compared these two peptides with respect to secondary structure, in vivo bacteriocin (antagonistic) activity, ability to permeabilize cell membranes, and pheromone activity, to gain insight into how PlnA functions as a bacteriocin and as a pheromone. The two enantiomers are expected to assume equivalent, but mirror-image, conformations. Consequently, the D-enantiomeric form should be active if a nonchiral interaction (with, for instance, the lipids in the cell membrane) is sufficient for PlnA to function, whereas it should be inactive if an interaction with a chiral component (for instance, a membrane-associated protein receptor) is required. Moreover, comparison of the bacteriocin and the pheromone activities of PlnA-22L and PlnA-22D indicates to what extent these two activities are coupled.

MATERIALS AND METHODS

Synthesis, Purification, and Analysis of Peptides. PlnA-22L and PlnA-22D were synthesized according to the sequence reported previously (8, 9). For purification, using the FPLC chromatography system (Pharmacia Biotech, Uppsala, Sweden), the synthesized peptides were solubilized in 0.1% (v/v) trifluoroacetic acid and applied to a PepRPC HR 5/5 C₂/C₁₈ reverse-phase column (Pharmacia Biotech) equilibrated with 0.1% (v/v) trifluoroacetic acid. The peptides were eluted from the reverse-phase column with a linear 15–60% (v/v) 2-propanol gradient containing 0.1% (v/v) trifluoroacetic acid. The chromatography fractions containing the desired peptides were then diluted 4–5-fold with H₂O containing 0.1% (v/v) trifluoroacetic acid and rechromatographed on the reverse-phase column. This was repeated 2–3 times until homogeneous fractions of the desired peptides were obtained. The primary structures and purity of the peptides were confirmed by protein sequencing (Applied Biosystems automatic sequencer with an on-line 120A phenylthiohydantion amino acid analyzer), by mass spectroscopy analysis (HP 2025A MALDI-TOF), by analytical reverse-phase chromatography using a μ RPC SC 2.1/10 C₂/C₁₈ column on the SMART-system (Pharmacia Biotech), and by capillary electrophoresis.

Bacterial Strains and Media. The bacteriocin-producing strain used in this study was *L. plantarum* C11 (17, 18). Cells with a stable Bac[−] phenotype were obtained as described previously (3). In such cells, the Bac⁺ phenotype can be restored by adding PlnA as described previously (3, 8). *L. plantarum* C11 was grown at 30 °C in MRS broth (Oxoid).

The following bacteria were used as indicator cells when assaying PlnA, PlnE/F, and PlnJ/K for bacteriocin activity: *Lactobacillus plantarum* 965, *Lactobacillus casei* NCDO 2713, *Lactobacillus sake* NCDO 2714, *Lactobacillus viridescens* NCDO 1655, *Pediococcus pentosaceus* NCDO 990, and *Carnobacterium piscicola* U149. They were all grown at 30 °C in MRS broth.

Lactobacillus sake NCDO 2714 was used for Δ pH and $\Delta\psi$ measurements.

Bacteriocin Assay. Bacteriocin activity was determined essentially as described earlier (19), using a microtiter plate assay. Each well of the plate contained 200 μ L of MRS broth (Oxoid), bacteriocin fractions at 2-fold dilutions, and a suitable indicator organism, OD₆₀₀ = 0.001. The microtiter plate cultures were incubated for 12–15 h at 30 °C, after which growth inhibition of the indicator organism was measured spectrophotometrically at 600 nm by use of a Dynatech microplate reader. When this assay system is standardized with respect to the amount of indicator used and the incubation time and temperature, determination of bacteriocin activity was reproducible to within a 2-fold dilution. One bacteriocin unit was defined as the amount of bacteriocin that inhibited growth of the indicator organism by 50% (50% of the turbidity of the control culture without bacteriocin).

Protonmotive Force Measurements. Generation of transmembrane electrical potential ($\Delta\psi$) was measured with the fluorescent probe [DiSC₃(5)] (20). Cells (*Lactobacillus sake* NCDO 2714) were used only directly after isolation. The transmembrane pH gradient (Δ pH) was measured by loading the cells with the fluorescent pH indicator BCECF as described previously (21).

PlnA-Induced Bacteriocin Production. An overnight stationary culture of *L. plantarum* C11 Bac[−] was diluted 1 to 1000 with MRS broth and grown for 8 h (to OD₆₀₀ 0.04) prior to the addition of PlnA (the amount of PlnA used is indicated in the figures). The transformation of the Bac[−] state to the bacteriocin-producing Bac⁺ state upon adding PlnA was then determined by following bacteriocin production with time, using the bacteriocin assay described above and the indicator strain *L. plantarum* 965 (which is sensitive to both PlnE/F and PlnJ/K).

Determination of Peptide Concentration. Absorbance at 280 nm was measured, and the peptide concentration was calculated using the molar extinction coefficient at 280 nm deduced from the amino acid composition of the peptides to be 12 660 M^{−1} cm^{−1} in 6.0 M guanidium hydrochloride, 0.02 M phosphate buffer, pH 6.5.

Liposome Preparation. Single-bilayer phospholipid vesicles were prepared essentially according to the procedure of Batzri and Korn (22). Eight micromoles of dioleoyl-L- α -phosphatidyl-DL-glycerol (Ole₂GroPGro, Sigma) or dioleoyl-L- α -phosphatidylcholine (Ole₂GroPCho, Sigma) dissolved in chloroform was carefully dried under a stream of ultrapure nitrogen. The dried lipids were redissolved in 1 volume of absolute ethanol and dried again. Subsequently, the lipids were redissolved in 200 μ L of absolute ethanol and slowly (ca. 100 μ L/min), and at constant speed, injected into 4 mL of 20 mM sodium phosphate (pH 5.3) at room temperature. Ethanol was removed by dialysis against 20 mM sodium phosphate (pH 5.3).

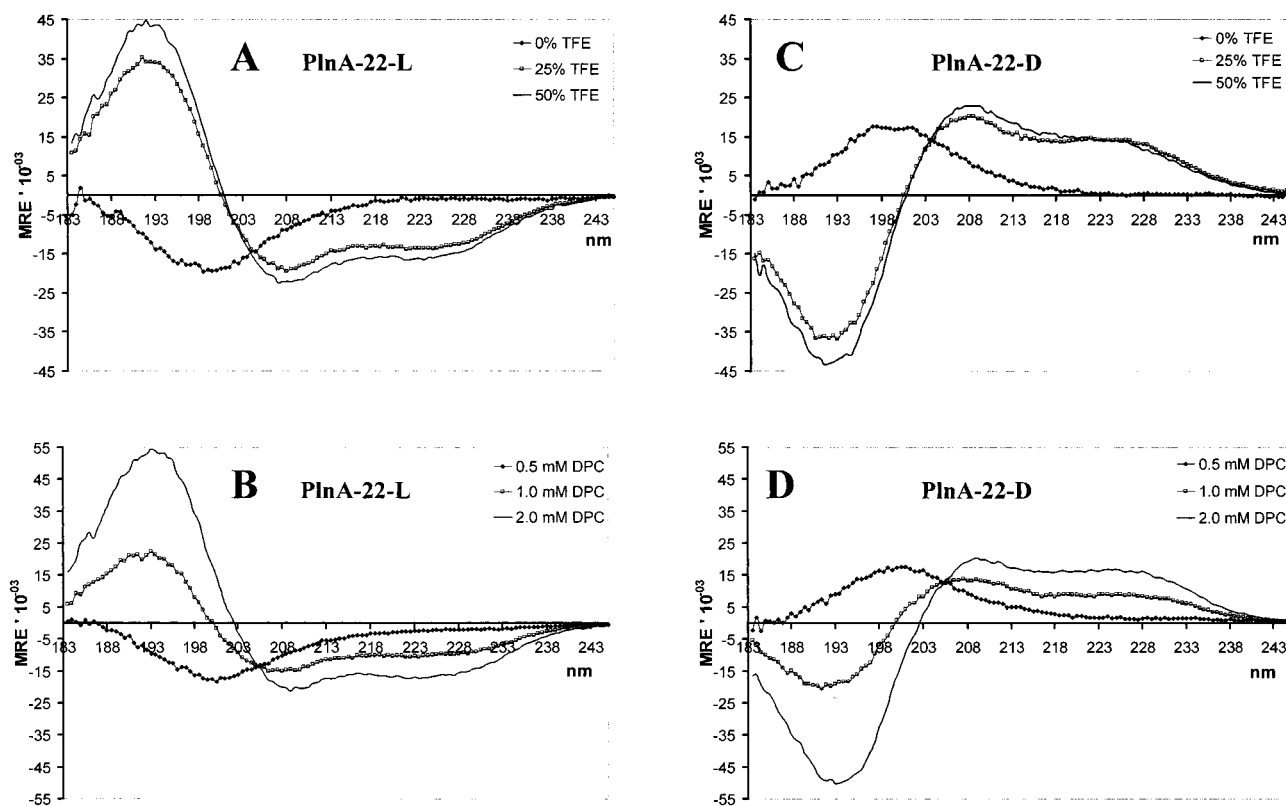


FIGURE 1: Circular dichroism spectra of the L- and D-enantiomers of PlnA in the presence of trifluoroethanol and dodecylphosphocholine. PlnA L-enantiomer titrated with (A) trifluoroethanol and (B) dodecylphosphocholine. PlnA D-enantiomer titrated with (C) trifluoroethanol and (D) dodecylphosphocholine. All measurements were done with a protein concentration of 0.15 mg/mL in 20 mM sodium phosphate, pH 5.3.

Circular Dichroism. CD spectra were recorded using a Jobin-Yvon autodichrograph Mark IV spectropolarimeter calibrated with epianthosterone. Measurements were performed at 25 °C, using a quartz cuvette with a path length of 0.05 cm and a peptide concentration of 0.15 mg/mL in 20 mM sodium phosphate (pH 5.3). Some measurements were done using a 0.5 cm cuvette and a peptide concentration of 0.0075 mg/mL in order to investigate the concentration dependency of structure induction. For all the conditions used in this study, structure induction was independent of peptide concentration.

Samples were scanned 4–8 times at 20 nm/min with a time constant of 2 s and a slit width of 2 nm, usually over the wavelength range 183–245 nm. The data were averaged, and the spectrum of a protein-free control sample was subtracted. The α -helical contents of PlnA-22L under the varying solvent conditions were calculated after smoothing, from the mean residual ellipticity at 222 nm ($[\theta]_{222}$) using the formula:

$$f_H = [\theta]_{222} / [-40000(1 - 2.5/n)]$$

where f_H represents the α -helical content and n represents the number of peptide bonds (23). To make the same formula applicable to PlnA-22D, θ values were first multiplied by -1 . All measurements were conducted at least twice. Crucial measurements were repeated several times until standard deviations in the percentage helicity were below 2%.

RESULTS

Membrane-like Entities Induce Equivalent but Mirror-Image Helical Structures in PlnA-22L and PlnA-22D. PlnA-

22L and its D-enantiomer (PlnA-22D) were synthesized according to the amino acid sequence of PlnA, their sequence being Tyr-Ser-Leu-Gln-Met-Gly-Ala-Thr-Ala-Ile-Lys-Gln-Val-Lys-Lys-Leu-Phe-Lys-Lys-Trp-Gly-Trp (the untruncated 26-mer of PlnA contains four more residues at the N-terminus: Lys-Ser-Ser-Ala; 9). After subsequent purification by reverse-phase chromatography, the primary structure and purity of the synthesized peptides were confirmed by protein sequencing and mass spectroscopy. The CD spectra of PlnA-22L and PlnA-22D in aqueous solution (pH 5.3) revealed that the peptides had little, if any, helical content (Figure 1).

Trifluoroethanol is known to induce and stabilize α -helical structure in peptides that have an intrinsic tendency to adopt this type of secondary structure (24–26). CD measurements showed that trifluoroethanol induced helical structure in both PlnA-22L and PlnA-22D at relatively low trifluoroethanol concentrations (Figure 1). Already at a concentration of 25%, trifluoroethanol induced a helical content of approximately 40% (calculated from the spectra in Figure 1A,C). Only a slight increase in the helical content (40–45% helical content) was obtained upon increasing the trifluoroethanol concentration to 50% (Figure 1A,C). Increasing the trifluoroethanol concentration to 65% did not significantly increase the helical content (spectra not shown). The spectra of PlnA-22L and PlnA-22D had opposite signs, but were otherwise equivalent (Figure 1A,C), indicating that the two enantiomers have mirror-image conformations.

Membrane-like entities, such as micelles and liposomes, also induced helical structure in both the PlnA L- and D-enantiomers. CD spectra of the peptides exposed to

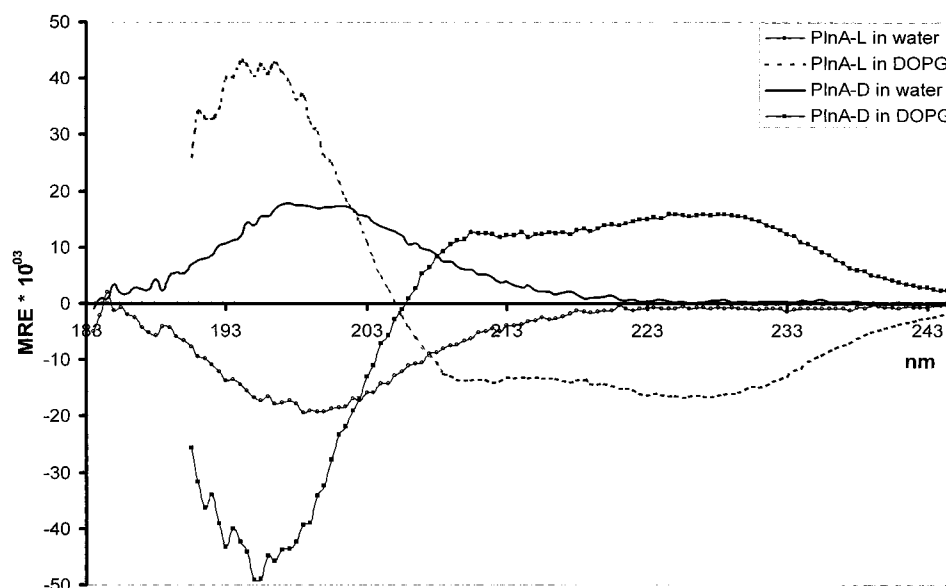


FIGURE 2: Circular dichroism spectra of the L- and D-enantiomers of PlnA-22 in water and in 1.4 mM Ole₂GroPGro. All measurements were conducted using a peptide concentration of 0.15 mg/mL in 20 mM sodium phosphate buffer, pH 5.3.

dodecylphosphocholine at concentrations ranging from 0.5 mM [which is below the critical micelle concentration of about 1 mM (27)] to 4 mM (which is above the critical micelle concentration) revealed that helical structure was induced in both the L- and D-enantiomers of PlnA once the dodecylphosphocholine concentrations exceeded the critical micelle concentration (Figure 1B,D). Maximal helical content (45–50% helical content, calculated from the spectra in Figure 1B,D) was attained already at a concentration of 2 mM dodecylphosphocholine, immediately after exceeding the critical micelle concentration. The spectra obtained with 4 mM dodecylphosphocholine did not differ significantly from those obtained with 2 mM (results not shown). At dodecylphosphocholine concentrations of 0.5 mM, the helical content was nearly identical to that in aqueous solution (less than 1–2%), whereas near the critical micelle concentration (1 mM) a transition state was observed where the helical content (approximately 25%) was between that in pure water and the maximum content obtained in 2 mM dodecylphosphocholine (Figure 1B,D). Similar to the results obtained with trifluoroethanol, the spectra of PlnA-22L and PlnA-22D in dodecylphosphocholine had opposite signs, but were otherwise equivalent (Figure 1B,D), indicating that the structures of the two peptides are mirror images.

Anionic Ole₂GroPGro liposomes at a lipid-to-peptide ratio of approximately 20:1 induced a helical content of 40–45% (calculated from the spectra in Figure 2) in both of the PlnA-22 enantiomers. At this lipid-to-peptide ratio, maximum structure induction was obtained, as illustrated by the fact that increasing the lipid-to-peptide ratio 20-fold had no effect on the CD spectra (data not shown). Thus, anionic liposomes induced approximately the same amount of helical structure as was observed in 25–50% trifluoroethanol and in the presence of dodecylphosphocholine micelles. In contrast to the anionic Ole₂GroPGro liposomes, zwitterionic Ole₂GroPCho liposomes induced only slightly more helical structure (about 7% helical content) than that obtained in aqueous solution (data not shown). The spectra obtained in the presence of liposomes again show that the structures of the two PlnA-22 enantiomers are mirror images. PlnA-22L

Table 1: Minimum Inhibition Concentrations (nM) for PlnA-22-L and PlnA-22-D^a

indicator strain	PlnA enantiomer	
	plantaricin A L-form	plantaricin A D-form
<i>Lactobacillus plantarum</i> 965	300	300
<i>Lactobacillus casei</i> NCDO 2713	$\geq 1 \times 10^4$	$\geq 1 \times 10^4$
<i>Lactobacillus sake</i> NCDO 2714	20	30
<i>Lactobacillus Viridescens</i> NCDO 1655	10	20
<i>Pediococcus pentosaceus</i> NCDO 990	$\geq 1 \times 10^4$	$\geq 1 \times 10^4$
<i>Carnobacterium piscicola</i> U149	$\geq 1 \times 10^4$	$\geq 1 \times 10^4$

^a The values in the table are concentrations (nanomolar) of peptides that caused 50% growth inhibition of the indicator strains in the bacteriocin assay.

is expected to adopt a normal right-handed α -helix, whereas the helical structure adopted by PlnA-22D is expected to be left-handed.

A Nonchiral Interaction Is Sufficient for PlnA To Function as a Bacteriocin. A strain-specific bacteriocin activity (antagonistic activity) was detected at nanomolar concentrations when PlnA-22L and PlnA-22D were screened against seven different indicator strains (Table 1). For all indicator strains tested, the two PlnA enantiomers had the same antagonistic potency, indicating that the bacteriocin activity of PlnA does not depend on a chiral interaction.

Both PlnA Enantiomers Permeabilize Cell Membranes. Both PlnA-22L and PlnA-22D effectively dissipated the transmembrane pH gradient (Δ pH; Figure 3A,B). Also the transmembrane electrical potential ($\Delta\psi$) generated by addition of valinomycin to cells suspended in Na-Pipes was effectively dissipated by both enantiomers (Figure 3C,D). They also dissipated the $\Delta\psi$ generated by cells suspended in potassium phosphate in the presence of 0.5% glucose, and after the conversion of Δ pH into $\Delta\psi$ by nigericin (data not shown). Immediately after the addition of only 60 nM of either the L- or the D-enantiomer of PlnA, there was a rapid, but incomplete, dissipation of the protonmotive force (Figure 3).

A Chiral Interaction Is Required for PlnA To Function as a Pheromone. PlnA can induce the expression of a number

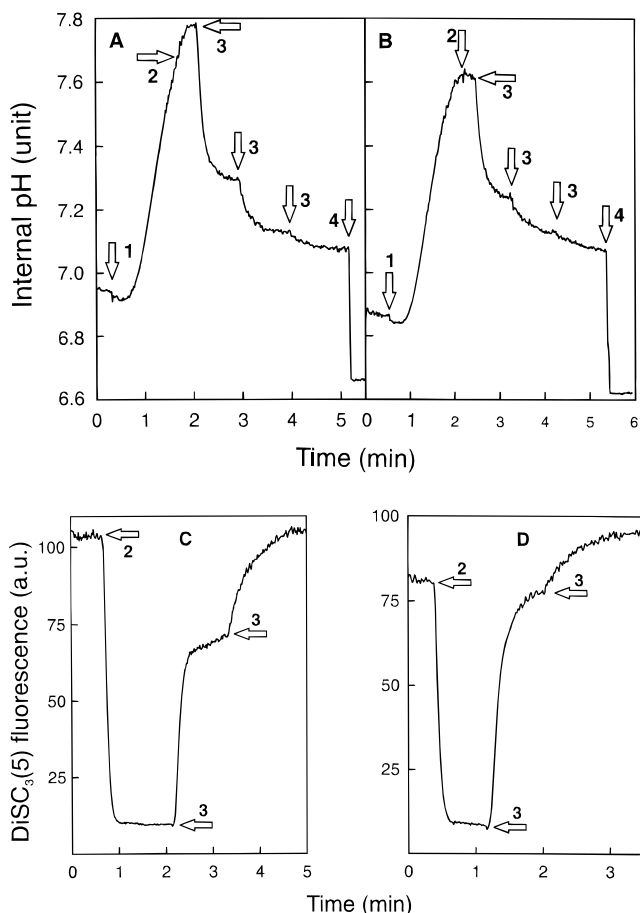


FIGURE 3: Dissipation of ΔpH by PlnA-22L (A) and PlnA-22D (B). Cells ($30 \mu\text{g}$ of protein/mL), loaded with BCECF, suspended in 50 mM potassium phosphate, pH 6.5, were energized with 0.5% glucose (arrows 1). After addition of valinomycin ($0.2 \mu\text{M}$, arrows 2), that converts the $\Delta\psi$ into a ΔpH , PlnA-22L (A) or PlnA-22D (B) was added at 60 nM at each arrow 3. Nigericin ($0.2 \mu\text{M}$, arrows 4) caused full dissipation of the ΔpH . (C, D) Dissipation of $\Delta\psi$ by PlnA-22L (C) and PlnA-22D (D). To cells ($30 \mu\text{g}$ of protein/mL) suspended in 50 mM Na-Pipes, pH 7.0, valinomycin ($0.2 \mu\text{M}$, arrows 2) was added to induce a $\Delta\psi$. Addition of PlnA-22L (C) or PlnA-22D (D) at 60 nM at the arrows 3 dissipates the $\Delta\psi$. Addition of solvent without PlnA had no effect on ΔpH or $\Delta\psi$.

of genes in *L. plantarum* C11, among which are the genes encoding the two peptide bacteriocins, PlnE/F and PlnJ/K (3, 8, 10). The pheromone activity of PlnA may therefore be determined by measuring the bacteriocin activity that is secreted by *L. plantarum* C11 when it is converted from the Bac^- to the Bac^+ state upon exposure to PlnA. *L. plantarum* 965 was chosen as indicator organism when quantitating the amount of bacteriocin induced by PlnA, since this indicator is approximately 3000 times more sensitive to PlnJ/K than to PlnA (8). Thus, the bacteriocin activity of induced PlnJ/K may be measured under conditions where the PlnA peptides which were added as inducing factors do not contribute to the bacteriocin activity.

Figure 4 shows that PlnA-22L could induce bacteriocin production in *L. plantarum* C11 and that the amount of bacteriocin produced was highly dependent on the amount of pheromone used. Bacteriocin levels reached a clear peak at about 5–6 h after addition of PlnA-22L. In contrast, PlnA-22D was not capable of inducing bacteriocin production, not even at the highest concentrations tested (800 nM,

being 400 times higher than the lowest concentration of PlnA-22L giving detectable bacteriocin production; Figure 4). However, an excess of the D-enantiomer inhibited the pheromone activity of the L-enantiomer: a 100- and 500-fold excess of the D-enantiomer inhibited the pheromone activity of the L-enantiomer (at a concentration of 20 nM) by, respectively, 80 and 99%.

DISCUSSION

The CD studies with trifluoroethanol and dodecylphosphocholine micelles show that the naturally occurring PlnA-22L has an intrinsic tendency to form a right-handed α -helix. As shown in Figure 5, this helix is expected to be highly amphiphilic. Dodecylphosphocholine induced structure only at concentrations above its critical micelle concentration, indicating that peptide interaction with micelles, rather than with free dodecylphosphocholine molecules, induced structure. The studies with liposomes show that an α -helix is also formed in the presence of membranes. Interestingly, whereas negatively charged membranes induced as much as 45% helicity, zwitterionic membranes induced only $\sim 7\%$ helicity. This observation is consistent with the hypothesis that electrostatic interactions between positively charged residues on the peptide and negatively charged groups in the membrane are important for structuring and functionality of PlnA. An alternative explanation for the observed dependence of structure induction on charge is that electrostatic repulsion of neighboring molecules in negatively charged liposomes creates a less densely packed surface into which the peptides may more easily insert. Taken together, the CD studies show that PlnA resembles bacteriocins in that it forms an amphiphilic α -helix in the presence of membrane-mimicking entities or negatively charged membranes (28–30).

The CD spectra obtained for PlnA-22D were in all cases mirror images of the spectra obtained for PlnA-22L. Thus, PlnA-22D forms a left-handed amphiphilic α -helix in the presence of membrane-mimicking entities or negatively charged membranes. These results indicate that comparative studies of the functionality of PlnA-22L and PlnA-22D may be used to investigate whether PlnA-22 functions through chiral interactions. There are several examples of analogous studies where D-enantiomers have been used to characterize the chirality of peptide–receptor interactions. For peptide hormones (such as angiotensin, oxytocin, and bradykinin) that interact with chiral receptors, the D-enantiomers are biologically inactive (31–33). In contrast, the D-enantiomers of the antimicrobial peptides (cecropin, magainin, and melittin) display the same antimicrobial activity as the naturally occurring L-forms, indicating that the functioning of these peptides does not depend on chiral interactions (34).

The D-enantiomer of PlnA-22 had the same strain-specific bacteriocin activity as the naturally occurring L-enantiomer. Both enantiomers permeabilized the target membrane, causing (partial) dissipation of the ΔpH and $\Delta\psi$. Lactococcin G, which like PlnA forms amphiphilic α -helices in the presence of membrane-mimicking entities or liposomes, has also been shown to permeabilize target cell membranes (28, 35). The strain-specific bacteriocin activity of the small amphiphilic α -helical PlnA peptides is clearly mediated through interactions with nonchiral cell components (possibly

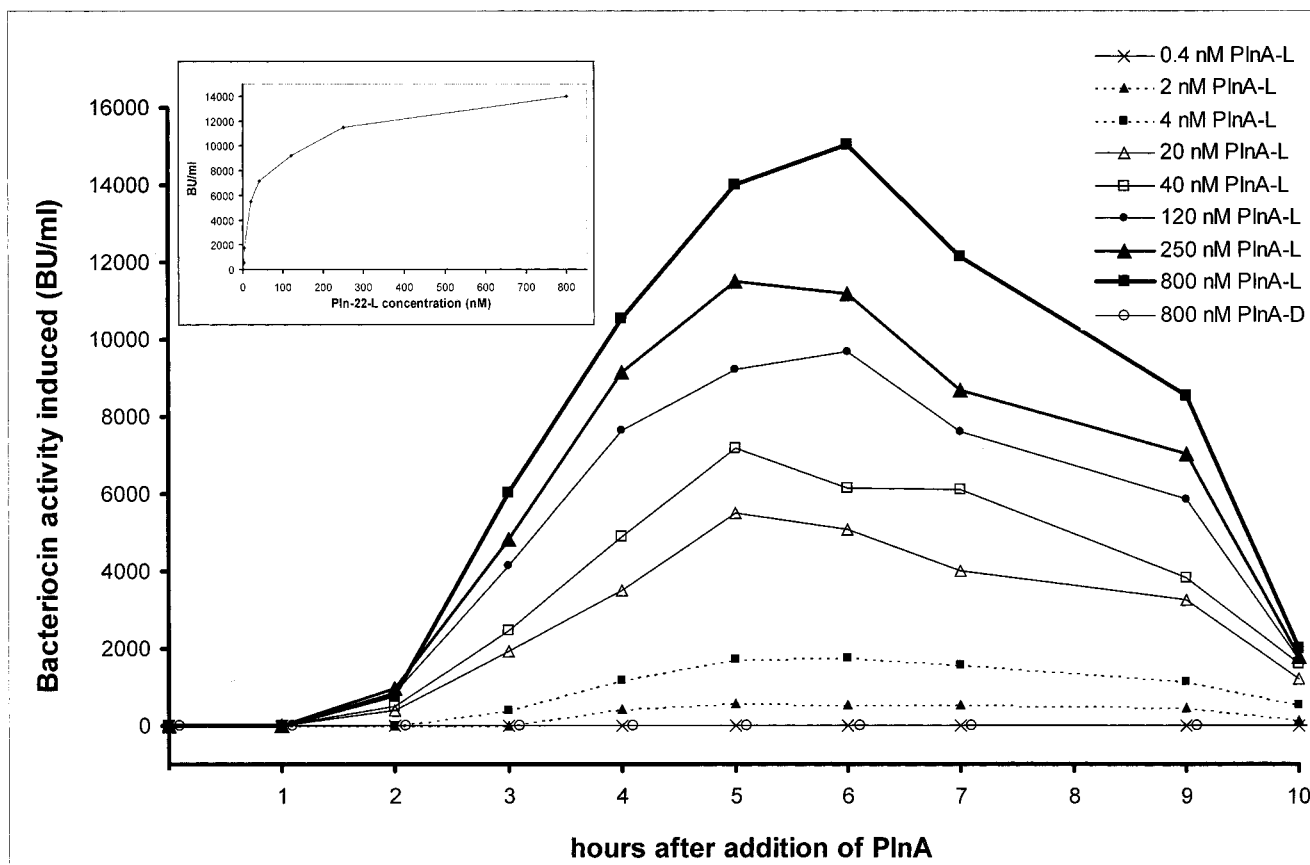
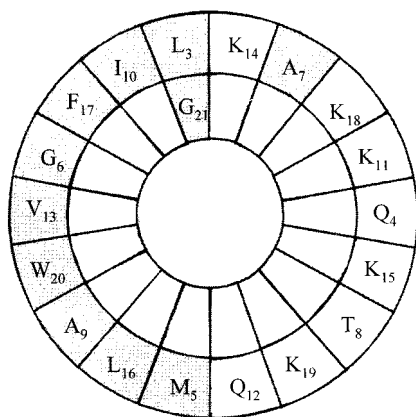


FIGURE 4: Pheromone-induced bacteriocin production by *L. plantarum* C11 as a function of the time passed after adding various amounts of PlnA-22L or PlnA-22D. The inset shows PlnA-induced (6 h after adding PlnA-22L) bacteriocin production as a function of the amount of PlnA-22L added.



activity may simply be due to the fact that the most sensitive target strains have yet to be identified. Moreover, there are other examples of peptides from lactic acid bacteria that act both as bacteriocins and as pheromones [nisin and carnobacteriocin B2 (5, 13, 37–39)]. An alternative interpretation is that the bacteriocin activity is an evolutionary remnant: PlnA may be an evolutionary intermediate between the inducing factors with potent bacteriocin activity (such as nisin and carnobacteriocin B2) and the very short inducing factors with no bacteriocin activity, such as the 19-mer peptide which induces the production of the bacteriocin sakacin P (4, 7).

ACKNOWLEDGMENT

We thank Bjørg Egelanddal at The Norwegian Food Research Institute, Ås, Norway, for making a CD spectrometer available to us.

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BI981532J